BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Rh(D) Antigen Expression and Isolation of a New Rh(D) cDNA Isoform in Human Erythroleukemic K562 Cells

By Kimita Suyama, Ruth Lunn, St phanie Haller, and Jack G ldstein

Human erythroleukemic K562 cells are known to have several erythroid properties. K562 cells possess Rh mRNAs, but expression of Rh proteins has not previously been reported. We immunoprecipitated Rh protein from K562 cell lysate using rabbit anti-Rh and detected Rh(D) antigens on K562 cells using fluorescence-actlvated cell sorting (FACS). These results suggest that K562 cells will be useful as an expression model for most Rh antigens. We also cloned a new Rh(D) cDNA isoform (RhK562-II), from a K562 cDNA library using polymerase chain reaction (PCR) with 5' and 3' end oligonucleotides of the published Rh(e/E) antigen encoding cDNA sequence as primers. Sequence analysis showed that RhK562-II is composed of 951 nucleotides (316 amino acids), identical to the first 939 nucleotides (exons 1 to 6) of one of the Rh(D) cDNAs (RhXIII), xcept for nucleotide 654 (C → G exchange). How ver, this exchang is the sam as that of another published Rh(D) cDNA (RhPII cDNA). RhK562-II is deprived of exons 7 and 8 (nucleotides 940 to 1,153), followed by an identical sequence up to the 3' end of the open-reading frame of the RhXIII cDNA, which causes a frame-shift mutation and produces a premature stop codon. In vitr expression of RhK562-II using the transcription and translation rabbit reticulocyte lysate system produced two major Rh-related proteins (30 kD and 25 kD), which were immunoprecipitated by rabbit polyclonal anti-Rh and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

THE RH BLOOD GROUP antigens, especially Rh(D), are of paramount importance in transfusion medicine, ranking second only to the ABO system. These antigens (C/c, D, E/e) are carried by distinct but highly homologous unglycosylated, fatty-acylated proteins that share an identical N-terminal amino acid sequence.[1-5] Rh antigen expression occurs early in hematopoietic differentiation, being detected in colony-forming unit–erythroid (CFU-E) cells, but not burst-forming unit–erythroid (BFU-E) cells.[6] However, they have not been detected in human erythroleukemic K562 cells, which are at the proerythroid stage of erythroid development, even though Rh mRNA is present.[7]

Further elucidation of the structure and antigenicity of these Rh proteins can be facilitated by molecular analysis. To date, the genes encoding several Rh antigens and an Rh-related glycosylated protein that might be important in Rh antigenicity have been cloned. Two research groups have independently isolated and sequenced a cDNA gene from a human bone marrow library specific for the Rh(E) or (e) antigen.[7,8] The predicted translated product of this gene is a 417–amino acid protein with a molecular weight of 45.5 kD; however, this protein migrates anomalously on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (33 kD) due to its high hydrophobicity.[9] Other reported Rh cDNA clones include Rh(D) from a human bone marrow library[10] and two Rh-related clones from cultured erythroid cells.[11] We recently reported transient expression of a Rh cDNA clone in COS-1 cells.[12]

Genomic studies by Colin et al[13] support the two-locus model of Rh inheritance. According to this model, Rh-positive individuals have two Rh polypeptide genes, whereas only one gene is found in Rh-negative individuals, which suggests that one gene encodes for Rh(D) and the other Rh(C/c) and Rh(E/e). Although there appear to be only two Rh genes, the Rh blood group system is extremely polymorphic. The discovery of several Rh mRNAs from a human bone marrow library that are derived from alternative splicing of the gene encoding C/c and E/e provides an explanation of Rh diversity from one gene.[14]

We describe here the isolation of a new Rh cDNA clone from a human erythroleukemic K562 library, which is a splicing isoform of the gene encoding the Rh(D) antigen. In addition, we report the expression of Rh polypeptides in K562 cells. Thus, K562 cells may provide a model system to study Rh antigenicity, since both polymorphisms and protein expression can be studied. Following submission of this report, a report has appeared that describes two Rh(D) variants associated with red blood cells.[15]

MATERIALS AND METHODS

Materials. Human erythroleukemic K562 cells were purchased from American Type Culture Collection (Rockville, MD), and K562 cDNA library from Clontech Laboratory (Palo Alto, CA). Restriction enzymes (*Eco*RI and *Bam*HI) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), LambdaSorb phage adsorbent from Promega (Madison, WI), and L-^{35}S-methionine, ^{32}P-labeled adenosine 5'-triphosphate, tetra (triethylammonium) salt was purchased from Dupont-NEN (Boston, MA). The sequenase 2.0 *kit* was obtained from USB, Cleveland, OH, and all cell culture media from Gibco-BRL, Grand Island, NY. Phenylmethylsulfonyl fluoride (PMSF), Trasylol, Protein A-Sepharose, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and fluorescein isothiocyanate (FITC)-labeled goat antihuman Ig were obtained from Sigma (St Louis, MO).

Fluorescence-activated cell sorting (FACS). Human erythroleukemic K562 cells (10^6 cells) were incubated with 0.2 mL polyclonal human anti-D (dilution 1:10) for 30 minutes at 4°C and washed three times in phosphate-buffered saline (PBS) with 0.1% NaN_3 and 0.5% bovine serum albumin (BSA). The cells were reincubated with FITC-labeled goat antihuman Ig for 30 minutes at 4°C, washed three times,

From the Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY.

Submitted October 22, 1993; accepted May 11, 1994.

Supported by Office of Naval Research Grant No. N00014-93-1-0364.

Address reprint requests to Jack Goldstein, PhD, New York Blood Center, 310 E 67 St, New York, NY 10021.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/94/8406-0009$3.00/0

and resuspended in PBS with 0.1% NaN_3 and 0.5% BSA. The cells were analyzed by FACS IV (Becton Dickinson, Sunnyville, CA). Data are presented as fluorescence intensity in fluorescence units versus cell frequency.

Immunoprecipitation of Rh proteins from K562 cells with rabbit polyclonal anti-Rh. Logarithmically growing K562 cells in complete RPMI media were collected and resuspended in RPMI without methionine. The cells (4×10^7 cells) were incubated with ^{35}S-methionine (200 μCi/mL) in 4 mL of the above media for 2 hours at 37°C. After washing three times with PBS, the cells were lysed by incubating with lysis buffer (0.25% SDS, 1% Triton X-100, 1 mmol/L TPCK, 1 mmol/L PMSF, and 100 U/mL Trasylol in 20 mmol/L Tris, pH 7.4) for 15 minutes at 4°C, and divided into four 1-mL aliquots. Supernatants collected by spinning were incubated with rabbit polyclonal anti-Rh (1:200 dilution) overnight at 4°C. Immune complexes extracted by Protein A-Sepharose were run on SDS-PAGE, followed by autoradiography.

Cloning of Rh cDNA by polymerase chain reaction (PCR). Preparation of template DNA from a human erythroleukemic K562 λgt 11 cDNA library was performed using the LambdaSorb phage adsorbent method. As primers, a 5′ end oligonucleotide with initiation codon (sense primer, 5′-GGATGAGCTCTAAGTACCCG-3′) sequence identical to that published for the Rh(e/E) cDNA clone, and a 3′ end oligonucleotide with a stop codon (antisense primer, 5′-CCCGGATCCTTAAAATCCAACAGCCAA-3′), were synthesized at the Microchemistry Laboratory of the New York Blood Center.

PCR amplification of DNA was performed using 0.05 mmol/L dNTP plus 0.75 mmol/L $MgCl_2$ in 10 mmol/L Tris-HCl, pH 9.0, with 50 mmol/L KCl and 0.1% Triton X-100. The reaction was performed in a MiniCycler (MJ Research, Watertown, MA) for 36 cycles, with each cycle consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes, and chain extension at 72°C for 3 minutes. PCR products were analyzed on 1% agarose gel. Southern blot analysis of the isolated cDNA was performed using a ^{32}P-labeled oligonucleotide (5′-GAGGGAACGGAGGATAAA-3′) specific for nucleotide 577 to 595 of the clone Rh(e/E) cDNA. The entire nucleotide sequence of the isolated cDNA was determined by the dideoxy-chain termination method using several oligonucleotides of the cloned Rh(e/E) cDNA, after subcloning the cDNA in pCRII vector (USB).

Expressions of Rh proteins in vitro. Expression of Rh proteins in vitro was performed with transcription- and translation-coupled reticulocyte lysate systems (Promega) according to the manufacturer's protocol and using Rh cDNA subcloned into pCRII as the expression vector [pCRIIRh(D) and pCRIIRhK562-II]. The reaction mixtures were incubated at 30°C for 90 minutes. Expressed Rh proteins were then immunoprecipitated with rabbit polyclonal anti-Rh or anti-RhNt[16] by incubating overnight at 4°C. The immune complexes were incubated with Protein A-Sepharose for 2 hours at 4°C, washed three times with lysis buffer and one time with 150 mmol/L NaCl in 50 mmol/L Tris, pH 6.8, eluted by boiling for 5 minutes in 1% SDS plus 8 mol/L urea plus 5% β-mercaptoethanol in 0.16 mol/L Tris, pH 6.8, and run on SDS-PAGE (12%), followed by autoradiography.

Determination of relative abundance of RhK562-I transcripts to native RhK562-II by reverse transcription coupled with PCR amplification. Total RNA was extracted from K562 cells using a RNA separation kit (Clontech). Briefly, total RNAs (1 μg) were incubated with oligo (dT) 12-18 primers at 70°C for 2 minutes. One tenth of the synthesized cDNAs were subjected to PCR amplification under the conditions described previously. PCR products were separated on 0.7% agarose gel and then transferred to Hybond-N+ (Amersham, Arlington Heights, IL) for Southern blot analysis. ^{32}P-labeled oligonucleotide (5′-GCAATCCTGCTGGACGGCTTC-3′) specific for nucleotide 271 to 291 of the cloned Rh(D) cDNA was used as a probe. After hybridization at 38°C overnight, Hybond-N+ was washed with 0.2× SSC (1× SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate) and 0.1% SDS for 30 minutes at 48°C as a final wash.

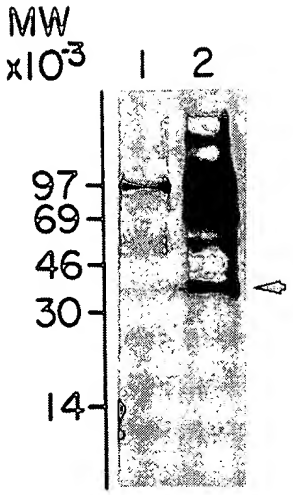

Fig 1. Autoradiogram of immunoprecipitates obtained from metabolically labeled K562 cells with ^{35}S-methionine using rabbit polyclonal anti-Rh. Details are described in the Methods. Lane 1, preimmune rabbit serum; lane 2, rabbit polyclonal anti-Rh. Arrow shows Rh protein region.

RESULTS

Rh antigen expression in human erythroleukemic K562 cells. Human erythroleukemic K562 cells have many erythroid properties, including the expression of red blood cell membrane proteins, such as glycophorin A.[17] In fact, Cherif-Zahar et al[7] detected Rh mRNA in these cells; however, expression of Rh protein has not been reported. To investigate Rh protein expression in K562 cells, we reacted a lysate prepared from K562 cells metabolically labeled with ^{35}S-methionine with a rabbit anti-Rh antibody. A Rh polypeptide band was found at the expected position (32 kD), as well as high–molecular weight polypeptides on SDS-PAGE (Fig 1, lane 2), while nonimmunized rabbit serum did not react with the large majority of these polypeptides (Fig 1, lane 1). These results suggest that Rh protein is expressed in K562 cells. The higher–molecular weight polypeptides could represent aggregates of the 32-kD Rh protein and/or Rh-specific or -nonspecific complexes. The Rh molecule is known to aggregate due to its extreme hydrophobicity. We have observed a similar pattern of high–molecular weight oligomers as depicted in Fig 1 in immunoprecipitates using our Rh-specific rabbit antibody from the lysates of COS-1 cells and rabbit recticulocytes transfected with Rh(D) cDNA (data not shown). Since neither of these nontransfected cells originally contain Rh proteins, the high-molecular coprecipitates cannot be due to Rh protein-glycoprotein complexes. On the other hand, immunoprecipitation of higher-molecular polypeptides with Rh-specific antibodies has been well documented. For example, Moore et al[18] reported the coprecipitation of 50-kD and 68-kD polypeptides, as well as the 32-kD Rh protein, from ^{125}I-labeled red blood cells using human anti-D plasma. Evidence for the existence of Rh protein-glycoprotein complexes in K562 cells is supported by the

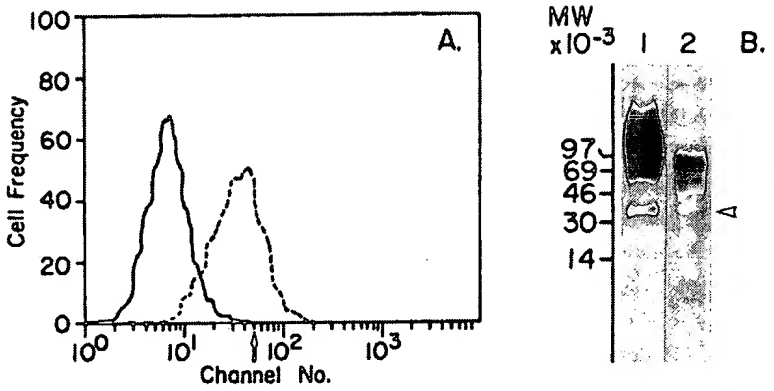

Fig 2. FACS profile of K562 cells in the presence of the human polyclonal anti-D and isolation of Rh(D) polypeptide from ^{125}I surface-labeled K562 cells by the same antibody. (A) K562 cells were incubated with a 1:10 dilution of either polyclonal anti-D or normal human type AB serum both containing 0.5% BSA and 0.1% NaN_3 in PBS. After washing, the cells were stained with FITC-conjugated goat antihuman Ig and analyzed by FACS. Details are described in the Methods. (——) Cells treated with control serum; (– – –) cells treated with human monoclonal anti-D. Arrow shows channel 50. (B) According to the method reported by Moore et al,[18] human red blood cells (R_2R_2) and K562 cells were first surface-labeled with ^{125}I, then sensitized with human polyclonal anti-D and solubilized. The immune complexes bound to Protein A-Sepharose were eluted and run on SDS-PAGE (12%). Lane 1, human red blood cells; lane 2, K562 cells. Arrow shows Rh(D) polypeptides.

isolation of the Rh glycoprotein in these cells (unpublished data). While the nature of these Rh-specific high–molecular weight oligomers cannot as yet be clearly defined, it seems to be characteristic of Rh immunoprecipitations.

To ascertain if expression occurred on the cell surface at this stage of erythroid development, we sensitized K562 cells with a human polyclonal anti-D, followed by an incubation with a second antibody (FITC-labeled antihuman Ig). These cells were analyzed by FACS (Fig 2A). K562 cells sensitized with anti-D exhibited a significant fluorescence intensity (channels 50 through 100) not observed with nonsensitized control K562 cells (channels 1 through 50) or with cells sensitized with anti-Fy^a Duffy, a blood group antigen not present on K562 cells (data not shown).[19] Fluorescence intensity was dependent on the concentration of anti-D in these cells, as well as in human red blood cells. Hence, K562 cells appear to express Rh(D) antigens on their surface.

While data shown in Fig 1 and Fig 2 demonstrate the presence of Rh polypeptides in K562 cell lysate and Rh(D) antigens on the cell surface, respectively, they do not definitively show that the polypeptides immunoprecipitated by rabbit anti-Rh are the same antigens detected on the cell surface by human anti-D. For this reason, we reacted ^{125}I surface-labeled K562 cells with human polyclonal anti-D. After solubilization, and extraction by Protein A-sepharose, we recovered a 32-kD band by SDS-PAGE (Fig 2B). These results clearly establish the presence of Rh(D) antigen-carrying proteins in K562 cells.

PCR amplification of Rh cDNA. We then set out to determine whether we could isolate cDNA clones for both the Rh acyl-proteins and the RhD50, which is a glycoprotein coprecipitated with human monoclonal anti-D, from a human erythroleukemic K562 library.[1] PCR amplification using 5′ and 3′ end–specific oligonucleotides for the RhD50 cDNA yielded a 1.2-kb clone whose sequence corresponded to that of the published RhD50 (data not shown). Two PCR products were obtained using Rh(e/E) or Rh IXb 5′ and 3′-specific primers: an expected 1.32-kb clone (RhK562-I cDNA) and an unknown 1-kb clone (RhK562-II cDNA), which may be a truncated gene of RhIXb. These bands were isolated and then rerun independently on a 1% agarose gel (Fig 3). Both of these clones bound strongly to a ^{32}P-labeled internal Rh(D)-specific oligonucleotide in Southern blot analysis (data not shown), which suggests that they were Rh-related cDNAs. Nucleotide sequencing using the dideoxy-chain termination method was implemented after subcloning each clone separately into PCRII vectors. The nucleotide sequence of RhK562-I (data not shown) was identical to the recently published Rh(D) cDNA clone (RhXIII) isolated from human bone marrow.[10]

Characterization of RhK562-II. RhK562-II was of great

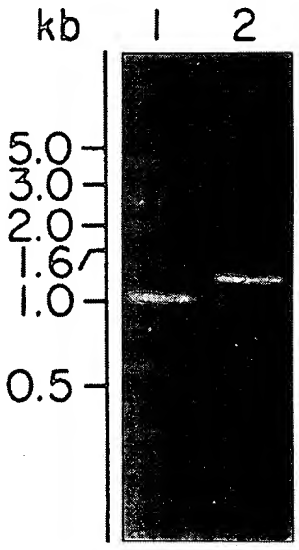

Fig 3. Cloning of a Rh cDNA from a human erythroleukemic cell (K562) cDNA library by PCR. Lanes 1 and 2, PCR products using 5′ end and 3′ end oligonucleotides of Rh cDNA as primers and K562 cell cDNA library as a template. Details are described in the Methods.

```
Met Ser Ser Lys Tyr Pro Arg Ser Val Arg Arg Cys Leu Pro Leu Trp Ala Leu Thr Leu      20
ATG AGC TCT AAG TAC CCG CGG TCT GTC CGG CGC TGC CTG CCC CTC TGG GCC CTA ACA CTG      60
Glu Ala Ala Leu Ile Leu Leu Phe Tyr Phe Phe Thr His Tyr Asp Ala Ser Leu Glu Asp      40
GAA GCA GCT CTC ATT CTC CTC TTC TAT TTT TTT ACC CAC TAT GAC GCT TCC TTA GAG GAT     120
Gln Lys Gly Leu Val Ala Ser Tyr Gln Val Gly Gln Asp Leu Thr Val Met Ala Ala Ile      60
CAA AAG GGG CTC GTG GCA TCC TAT CAA GTT GGC CAA GAT CTG ACC GTG ATG GCG GCC ATT     180
Gly Leu Gly Phe Leu Thr Ser Ser Phe Arg Arg His Ser Trp Ser Ser Val Ala Phe Asn      80
GGC TTG GGC TTC CTC ACC TCG AGT TTC CGG AGA CAC AGC TGG AGC AGT GTG GCC TTC AAC     240
Leu Phe Met Leu Ala Leu Gly Val Gln Trp Ala Ile Leu Leu Asp Gly Phe Leu Ser Gln     100
CTC TTC ATG CTG GCG CTT GGT GTG CAG TGG GCA ATC CTG CTG GAC GGC TTC CTG AGC CAG     300
Phe Pro Ser Gly Lys Val Val Ile Thr Leu Phe Ser Ile Arg Leu Ala Thr Met Ser Ala     120
TTC CCT TCT GGG AAG GTG GTC ATC ACA CTG TTC AGT ATT CGG CTG GCC ACC ATG AGT GCT     360
Leu Ser Val Leu Ile Ser Val Asp Ala Val Leu Gly Lys Val Asn Leu Ala Gln Leu Val     140
TTG TCG GTG CTG ATC TCA GTG GAT GCT GTC TTG GGG AAG GTC AAC TTG GCG CAG TTG GTG     420
Val Met Val Leu Val Glu Val Thr Ala Leu Gly Asn Leu Arg Met Val Ile Ser Asn Ile     160
GTG ATG GTG CTG GTG GAG GTG ACA GCT TTA GGC AAC CTG AGG ATG GTC ATC AGT AAT ATC     480
Phe Asn Thr Asp Tyr His Met Asn Met Met His Ile Tyr Val Phe Ala Ala Tyr Phe Gly     180
TTC AAC ACA GAC TAC CAC ATG AAC ATG ATG CAC ATC TAC GTG TTC GCA GCC TAT TTT GGG     540
Leu Ser Val Ala Trp Cys Leu Pro Lys Pro Leu Pro Glu Gly Thr Glu Asp Lys Asp Gln     200
CTG TCT GTG GCC TGG TGC CTG CCA AAG CCT CTA CCC GAG GGA ACG GAG GAT AAA GAT CAG     600
Thr Ala Thr Ile Pro Ser Leu Ser Ala Met Leu Gly Ala Leu Phe Leu Trp Met Phe Trp     220
ACA GCA ACG ATA CCC AGT TTG TCT GCC ATG CTG GGC GCC CTC TTC TTG TGG ATG TTC TGG     660
Pro Ser Phe Asn Ser Ala Leu Leu Arg Ser Pro Ile Glu Arg Lys Asn Ala Val Phe Asn     240
CCA AGT TTC AAC TCT GCT CTG CTG AGA AGT CCA ATC GAA AGG AAG AAT GCC GTG TTC AAC     720
Thr Tyr Tyr Ala Val Ala Val Ser Val Val Thr Ala Ile Ser Gly Ser Ser Leu Ala His     260
ACC TAC TAT GCT GTA GCA GTC AGC GTG GTG ACA GCC ATC TCA GGG TCA TCC TTG GCT CAC     780
Pro Gln Gly Lys Ile Ser Lys Thr Tyr Val His Ser Ala Val Leu Ala Gly Gly Val Ala     280
CCC CAA GGG AAG ATC AGC AAG ACT TAT GTG CAC AGT GCG GTG TTG GCA GGA GGC GTG GCT     840
Val Gly Thr Ser Cys His Leu Ile Pro Ser Pro Trp Leu Ala Met Val Leu Gly Leu Val     300
GTG GGT ACC TCG TGT CAC CTG ATC CCT TCT CCG TGG CTT GCC ATG GTG CTG GGT CTT GTG     900
Ala Gly Leu Ile Ser Val Gly Gly Ala Lys Tyr Leu Pro Val Cys Ser ***                 317
GCT GGG CTG ATC TCC GTC GGG GGA GCC AAG TAC CTG CCG GTT TGC TCC TAA                 951
```

Fig 4. DNA and the predicted protein sequence of RhK562-II cDNA. Box indicates the amino acid difference of the Rh(D) protein as a result of a single nucleotide difference (underlined).

interest to us because of its potential as a new Rh(D) cDNA-splicing isoform. Previously isolated truncated Rh clones have turned out to be Rh-splicing isoforms of the Rh(Cc/Ee)-specific gene.[13] Moreover, RhK562-I (which was isolated in the same PCR reaction as RhK562-II) was identical to Rh(D) cDNA; hence, there was a strong possibility that RhK562-II was an Rh(D) isoform. Thus, we characterized RhK562-II with respect to sequence analysis, membrane topology, and in vitro translation.

Sequence analysis of RhK562-II. Figure 4 depicts the complete nucleotide sequence of the 948-base open-reading frame clone, as well as the predicted 316 amino acids. Nucleotides 1 to 939 and 1,154 to 1,251 (data not shown) are identical to that of RhXIII, except for a C → G nucleotide exchange at position 654; however, this exchange may not be due to PCR error, since it is the same as another reported Rh cDNA clone.[20] A deletion occurs from nucleotides 950 to 1,153, causing a translation frame-shift that produces a premature stop codon at nucleotide 949 (Fig 5). Furthermore, this deletion corresponds to the reported intron/exon transitions, such that it encompasses exons 7 and 8. RhK562-II is an Rh isoform formed by alternative splicing of the Rh(D)-specific gene.

Membrane topology of RhK562-II. Figure 6 compares the membrane topology of the isoform RhK562-II based on the hydropathy analysis reported by Kyte et al[21] using a 6-amino acid window to that of the published prototype RhXIII.[11] The RhK562-II protein contains 10 transmembrane domains with both internal N- and C-termini, whereas the RhXIII protein has 13 transmembrane domains with an intracellular N-terminus. Although the carboxy end of RhXIII is predicted to be exofacial, biochemical studies have yielded contrasting results.[22]

With respect to possible epitope considerations in RhK562-II the following are of interest: (1) the exofacial cysteine (285) thought to be critical to Rh antigenicity is

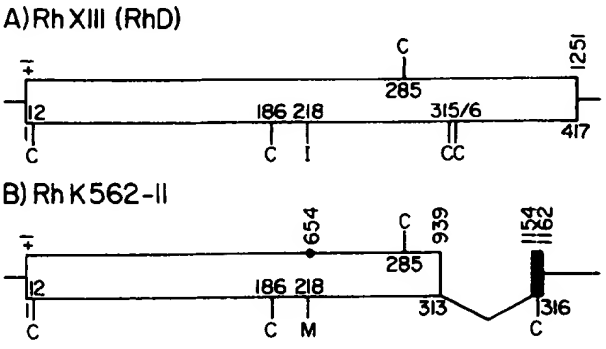

Fig 5. Comparison of RhK562-II to RhXIII (RhD) in nucleotide sequence and amino acid sequence. Open boxes represent coding sequences, and single lines indicate noncoding regions. Closed box indicates region of RhK562-II–encoding amino acids produced by frame-shift of RhXIII. Numbers below and inside boxes refer to amino acid positions starting from initiating methionine. Numbers above boxes indicate nucleotide positions starting from initiation codon. C, cysteine; M, methionine; I, isoleucine. Closed dot indicates the nucleotide difference (C → G).

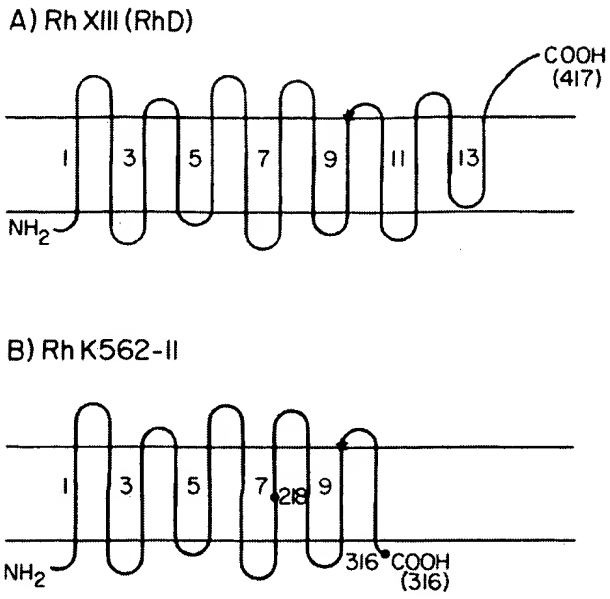

Fig 6. Comparison of membrane topology of the RhK562-II protein with that of RhXIII (RhD) protein. Membrane topology was predicted by hydropathy analysis according to Kyte et al[21] using a 6-amino acid window. Closed circles indicate amino acid polymorphisms. Stars indicate cysteine residue at 285.

conserved; (2) the N-terminus region containing a 17-kD chymotrypsin polypeptide, which is believed to be involved in Rh(D) antigen expression[16] is also conserved; and (3) the generation of a different C-terminus end due to the translational frame-shift mutation is unlikely to result in a new epitope, since it is intracellular and is only three amino acids long.

In vitro translation of RhK562-II. To ascertain if RhK562-II produced a viable protein recognized by Rh antibodies, we expressed this gene, as well as RhXIII, in a TNT transcription/translation-coupled reticulocyte lysate system. The expression vectors used in this system (pCRIIK562-II and pCRIIRhXIII) were constructed by subcloning each gene separately into pCRII vectors, which contain a T7 promoter. Rh antibodies used in the characterization of these translated proteins were a polyclonal rabbit anti-Rh that specifically binds to all Rh proteins, and a polyclonal rabbit anti-RhNt that recognizes the shared N-terminus of all Rh proteins. As shown in Fig 7, rabbit anti-Rh immunoprecipitated a 32-kD ^{35}S-methionine-incorporated protein produced by pCRIIRhXIII and two labeled proteins, an expected 30-kD, and a 26-kD produced by pCRK562-II. However, only the 30-kD band (Fig 7B) of the pCRIIK562-II translation products was immunoprecipitated with rabbit anti-RhNt. The smaller protein (26 kD) may be a product of a different initiation codon or a protease cleavage product in the N-terminal region.

We were interested in the expression of RhK562-II mRNA and the translated protein in K562 cells. As shown in Fig 1, a shortened Rh(D) polypeptide was not detected by immunoprecipitation using rabbit anti-Rh antibody. However, RhK562-II may exist as highly aggregated molecules and/or may exhibit abnormal behavior on SDS-PAGE due to its high hydrophobicity. This is supported by a report from Mouro et al[15] that Rh proteins from D^{VI}-type red blood cells, which lack exons 4, 5, and 6 of the Rh(D) gene, migrated as an unknown 69-kD protein, as well as 32-kD protein, on SDS-PAGE when tested by Western blot analysis using a rabbit antibody against the C-terminal peptide of Rh(D) proteins. However, we were able to determine the relative transcript abundance of RhK562-II to native RhK562-I in K562 cells using reverse-transcriptase PCR. Total RNAs were isolated from K562 cells, then cDNAs were synthesized from the RNAs by reverse transcriptase and the produced Rh(D) cDNAs were amplified by PCR using 5′ and 3′ end-specific primers of Rh(D) cDNA. PCR products were separated on a 0.7% agarose gel and transfered to Hybond-nylon. By Southern analysis, two bands, a 1.2-kb native RhK562-I and a 1.0-kb truncated RhK562-II, were detected using a nucleotide probe common to both native and truncated cDNAs (Fig 8). Relative abundance of RhK562-II to native RhK562-I was approximately 1:10.

DISCUSSION

Human erythroleukemic K562 cells may represent an alternative model for the study of Rh antigenicity, not only in terms of Rh expression in hematopoietic differentiation, but also for investigation of Rh(D) complex formation and potential function of Rh proteins and polymorphism of the Rh antigen system. This report of Rh(D) protein expression and the isolation of a new Rh(D) isoform in K562 cells provides credibility for this model.

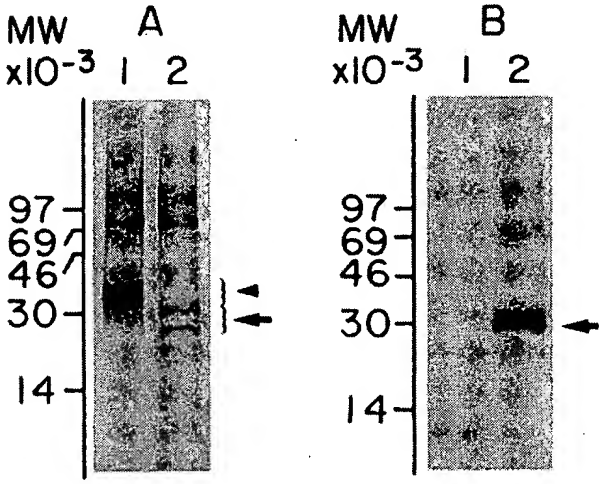

Fig 7. Expression of Rh cDNAs in a transcription/translation-coupled reticulocyte lysate system. (A) Autoradiogram of expressed Rh proteins obtained by immunoprecipitation with rabbit anti-Rh and separation on 12% SDS-PAGE. Lane 1, pCRIIRhXIII vector expression; lane 2, pCRIIRhK562-II vector expression. (B) Autoradiogram of expressed Rh proteins obtained by immunoprecipitation with rabbit anti-RhNt and separation on 12% SDS-PAGE. Lane 1, pCRII vector alone; lane 2, pCRIIRhK562-II vector. Details are described in the Methods. Arrowhead indicates Rh(D) protein region and arrow the RhK562-II protein region.

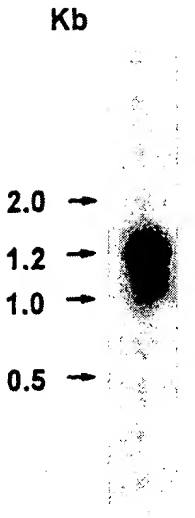

Fig 8. Relative abundance of RhK562-II transcripts to RhK562-I. Total RNAs were isolated from K562 cells and the cDNAs were produced by reverse transcriptase using the total RNAs, then RhK562-I cDNA and RhK562-II cDNA were specifically amplified by PCR using 5′ end and 3′ end primers of Rh(D) cDNA. They were separated on a 0.7% agarose gel and detected by Southern blot analysis using a probe common to both. Details are described in the Methods.

Although Rh mRNA has been detected in K562 cells, protein expression has not been reported.[7] Possible reasons for undetectable Rh antigens are that K562 cells simply produce considerably fewer antigen sites per cell and/or may have a slightly different configuration of Rh antigens on their cell surfaces, compared with red blood cells, and possibly insufficient titers of anti-Rh antibodies were used for detection. The former explanation is supported by the findings of Cherif-Zahar et al[7] that Rh mRNAs are present in lower amount in K562 cells compared with adult spleen erythroblasts. However, K562 cells clearly produced Rh antigen (Figs 1 and 2A and B). Our isolation of the RhD50 protein provides further evidence that K562 cells have some of the necessary Rh-related polypeptides thought to be required for Rh antigen expression.

K562 cells may also represent a means to study the generation of Rh polymorphism. Rh is the most polymorphic of the 19 known red blood cell group systems, consisting of at least 45 reported antigens.[23] Variations in Rh(D) expression can be both qualitative (partial D red blood cells) and quantitative (weak D red blood cells).[24] Qualitative variation in D is believed to be due to differences in expression of the number and type of the eight postulated overlapping D epitopes.

Possible mechanisms for the generation of these Rh(D) polymorphisms include mutations and alternative splicing. Since the epitopes appear to be overlapping, a single mutation could account for loss of several epitopes. Alternative splicing has been found to be a means to generate diversity in glycophorins C and D, which carry the Ge blood group system,[23] and Rh(C/c, E/e)-specific splicing isoforms have been isolated.[13] This report of a newly isolated Rh(D) isoform supports the model of alternative splicing as a significant mechanism of Rh diversity. Although this isoform was isolated from a human erythroleukemic K562 library, it is unlikely that it is merely the result of alternative expression that is developmentally regulated, since the prototype RhK562-I was also isolated from this same library. Moreover, three of the Rh(Cc/Ee) isoforms have been isolated from both human bone marrow and reticulocytes. Thus, we suspect that RhK562-II exists at different developmental stages. Epitope characterization of the expressed RhK562-II polypeptide will provide insight on how antigen diversity correlates with molecular RNA splicing.

ACKNOWLEDGMENT

We thank Jay Valinsky for his helpful advice, Harold Ralph for performing the FACS, and Tellervo Huima for her excellent photography.

REFERENCES

1. Agre P, Cartron JP: Molecular biology of Rh antigens. Blood 78:551, 1991

2. Hartel-Schenk S, Agre P: Mammalian red cell membrane Rh polypeptides are selectively palmitoylated subunits of a macromolecular complex. J Biol Chem 267:5569, 1992

3. Moore S, Woodrow CF, McClelland DBL: Isolation of membrane components associated with human red cell antigens Rh(D), (c), (E) and Fy^a. Nature 295:529, 1982

4. Gahmberg CG: Molecular identification of the human Rh(D) antigens. FEBS Lett 40:93, 1982

5. Blanchard D, Bloy C, Hermand P, Cartron JP, Saboori AM, Smith BL, Agre P: Two-dimensional iodopeptide mapping demonstrates that erythrocyte RhD, c and E polypeptides are structurally homologous but nonidentical. Blood 72:1424, 1988

6. Falkenburg FJH, Fibbe WE, van der Vaart-Duinkerken N, Nichols ME, Rubinstein P, Jansen J: Human erythroid progenitor cells express rhesus antigens. Blood 66:660, 1985

7. Cherif-Zahar B, Bloy C, Le Van Kim C, Blanchard D, Bailly P, Hermand P, Salmon C, Cartron JP, Colin Y: Molecular cloning and protein structure of a human blood group Rh polypeptide. Proc Natl Acad Sci USA 87:6243, 1990

8. Avent ND, Ridgwell K, Tanner MJA, Anstee DJ: cDNA cloning of a 30 kDa erythrocyte membrane protein associated with Rh (rhesus)-blood-group-antigen expression. Biochem J 271:821, 1990

9. Suyama K, Goldstein J, Aebersold R, Kent S: Regarding the size of Rh proteins. Blood 72:411, 1991

10. Le Van Kim C, Mouro I, Cherif-Zahar B, Raynal V, Cherrier C, Cartron JP, Colin Y: Molecular cloning and primary structure of the human blood group RhD polypeptide. Proc Natl Acad Sci USA 89:10925, 1992

11. Kajii E, Umenishi F, Iwamoto S, Ikemoto S: Isolation of a new cDNA clone encoding an Rh polypeptide associated with the Rh blood group system. Hum Genet 91:157, 1993

12. Suyama K, Roy S, Lunn R, Goldstein J: Expression of the 32-kd polypeptide of the Rh antigen. Blood 82:1006, 1993

13. Colin Y, Cherif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP: Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. Blood 78:2747, 1992

14. Le Van Kim C, Cherif-Zahar B, Raynal V, Mouro I, Lopez M, Cartron JP, Colin Y: Multiple Rh messenger RNA isoforms are produced by alternative splicing. Blood 80:1074, 1992

15. Mouro I, Le Van Kim C, Rouillac C, van Rhenen DJ, LePennec PY, Bailly P, Cartron JP, Colin Y: Rearrangements of the blood group RhD gene associated with the DVI category phenotype. Blood 83:1129, 1994

16. Suyama K, Goldstein J: Membrane orientation of Rh(D) polypeptide and partial localization of its epitope-containing domain. Blood 78:808, 1992

17. Rutherford TR, Clegg JB, Weatherall DJ: K562 human leukemic cells synthesize embryonic haemoglobin in response to haemin. Nature 280:164, 1979

18. Moore S, Woodrow CF, McClelland DBL: Isolation of membrane components associated with human red cell antigens Rh(D), (c), (E) and Fy^a. Nature 295:529, 1982

19. Chaudhuri A, Polyakova J, Zbrzezna V, Williams K, Gulati S, Pogo AO: Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor for the *Plasmodium vivax* malaria parasite. Proc Natl Acad Sci USA 90:10793, 1993

20. Arce MA, Thompson ES, Wagner S, Coyne KE, Ferdman BA, Lublin DM: Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. Blood 82:651, 1993

21. Kyte J, Doolittle RF: A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105, 1982

22. Avent ND, Butcher SK, Liu W, Mawby WJ, Mallinson G, Parsons SF, Anstee DJ, Tanner MJA: Localization of the C termini of the Rh (rhesus) polypeptides to the cytoplasmic face of the human erythrocyte membrane. J Biol Chem 267:15134, 1992

23. Tippett P: Serologically defined Rh determinants. J Immunogenet 17:247, 1990

24. Issitt PD: The Rh blood group system, 1988: Eight new antigens in nine years and some observations on the biochemistry and genetics of the system. Transfusion Med Rev 3:1, 1989